THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1985 by The American Society of Biological Chemists, Inc.

Vol. 260, No. 13, Issue of July 5, pp. 8070–8077, 1985
Printed in U.S.A.

Purification and Characterization of a Protein-Tyrosine Kinase Encoded by the Abelson Murine Leukemia Virus*

(Received for publication, November 13, 1984)

J. Gordon Foulkes‡§, Marie Chow‡¶, Carolyn Gorka‡, A. Raymond Frackelton, Jr.‖, and David Baltimore‡

From the ‡Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142 and the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and the ‖Department of Medicine, Roger Williams General Hospital and Brown University, Providence, Rhode Island 02908

Sequences termed v-*abl*, which encode the protein-tyrosine kinase activity of Abelson murine leukemia virus, have been expressed in *Escherichia coli* as a fusion product (p*tabl*50 kinase). This fusion protein contains 80 amino acids of SV40 small t and the 403 amino acid protein kinase domain of v-*abl*. We report here the purification and characterization of this kinase. The purified material contains two proteins (M_r = 59,800 and 57,200), both of which possess sequences derived from v-*abl*. Overall purification was 3,750-fold, with a 31% yield, such that 117 μg of kinase could be obtained from 40 g of *E. coli* within 6–7 days. The specific kinase activity is over 170 μmol of phosphate min^{-1} μmol^{-1}, comparable to the most active protein-serine kinases. Kinase activity is insensitive to K^+, Na^+, Ca^{2+}, Ca^{2+}-calmodulin, cAMP, or cAMP-dependent protein kinase inhibitor. The K_m for ATP is dependent on the concentration of the second substrate. GTP can also be used as a phosphate donor. The enzyme can phosphorylate peptides consisting of as few as two amino acids and, at a very low rate, free tyrosine. Incubation of the kinase with [γ-^{32}P]ATP results in incorporation of 1.0 mol of phosphate/mol of protein. This reaction, however, cannot be blocked by prior incubation with unlabeled ATP. Incubation of ^{32}P-labeled kinase with either ADP or ATP results in the synthesis of [^{32}P]ATP. This suggests the phosphotyrosine residue on the Abelson kinase contains a high energy phosphate bond.

The protein products of several RNA tumor virus transforming genes have been shown to contain regions that act as tyrosine-specific protein kinases (1, 2). Similar kinase activities are also associated with the membrane receptors for epidermal growth factor (EGF[1]) (3), platelet-derived growth factor (4, 5), insulin-like growth factor (6, 7), and insulin (8–11). These findings suggest that phosphorylation of certain proteins on tyrosine residues, both by oncogene products and by normal cellular growth factors, is involved in the control of cell growth. Although numerous phosphotyrosine-containing proteins have been observed in cells (1, 5, 12–14) and several proteins have been shown to act as substrates *in vitro* (8, 13–16), physiologically important targets for these enzymes have yet to be established in any system.

Previous studies in our laboratory have concentrated on the Abelson murine leukemia virus (A-MuLV), which encodes a transforming protein with tyrosine-specific kinase activity (17, 18). The gene encoding this protein is a fusion of coding sequences from the Moloney murine leukemia virus *gag* protein and a portion of the cellular c-*abl* gene, called v-*abl* (the oncogene of A-MuLV) (19). Systematic deletions of the v-*abl* gene have defined the minimum region required for both kinase activity and fibroblast transformation. These sequences encode a M_r = 45,000 protein (20).[2]

To gain further insight into the mechanism whereby A-MuLV induces cell transformation, we have transferred the DNA sequences encoding the minimal kinase domain of A-MuLV into a vector which allows expression in *Escherichia coli* (21). In this paper, we report a procedure for purifying this A-MuLV kinase to homogeneity, and present a characterization of its enzymatic properties.

EXPERIMENTAL PROCEDURES

Materials—DE52-cellulose and p81 phosphocellulose paper were purchased from Whatman. Hydroxylapatite, Affi-Gel Blue, SDS-polyacrylamide gel molecular weight markers, and silver stain for proteins were from Bio-Rad. Angiotensin II, ATP, ADP, GTP, kemptide (leu-Arg-Arg-Ala-Ser-Leu-Gly), Tris, ampicillin, and bovine serum albumin (M_r = 67,000) were obtained from Sigma. Except for bovine serum albumin, the other proteins used as molecular weight markers on gel filtration were obtained from Boehringer Mannheim and included cytochrome *c* (horse heart, M_r = 13,370), peroxidase (horseradish, M_r = 43,000), alcohol dehydrogenase (yeast, M_r = 143,000), and glutamate dehydrogenase (beef liver, M_r = 1,015,000, used for the determination of V_0). Yeast extract and tryptone were from Difco. Serine, tyrosine, Arg-Tyr, Lys-Tyr-Lys, Lys-Ser-Tyr, and Coomassie G-250 were from Serva. [γ-^{32}P]ATP and [γ-^{32}P]GTP were from ICN East Pharmaceuticals. Brij 35 (polyoxyethylene lauryl ether) and ethylene glycol were obtained from Fisher. PIPES and HEPES were both Ultrol grade from Calbiochem-Behring. Autoradiography was carried out using DuPont Cronex Xtra-Life intensifying screens and Kodak XAR-5 film. All other reagents were reagent grade or better.

Buffer Solutions—The following buffers were used during this work: Buffer A = 50 mM PIPES, pH 7.5, 0.1 mM EDTA, 0.015% Brij 35; Buffer B = 20 mM PIPES, pH 8.15, 0.1 mM EDTA, 0.015% Brij

* This work was supported by Program Project Grant CA 26717 from the National Cancer Institute. Part of this work was carried out while J. G. F. was a recipient of a Medical Research Council (United Kingdom) Travel Fellowship. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: National Institute for Medical Research, Mill Hill, London NW7 1AA, England, U. K.

¶ Present address: Department of Applied Biological Sciences, M.I.T., Cambridge, MA 02139.

[1] The abbreviations used are: EGF, epidermal growth factor; A-MuLV, Abelson murine leukemia virus; SDS, sodium dodecyl sulfate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] Prywes, R., Foulkes, J. G., and Baltimore, D. (1985) *J. Virol.*, in press.

35, 20% ethylene glycol, 0.2% β-mercaptoethanol; Buffer C = 20 mM Tris-HCl, pH 8.8 (0 °C), 0.015% Brij 35, 20% ethylene glycol, 0.2% β-mercaptoethanol, 100 mM NaCl; Buffer D = 50 mM PIPES, pH 7.5, 0.1 mM EDTA, 0.015% Brij 35, 10% ethylene glycol, 0.2% β-mercaptoethanol; Buffer E = 50 mM PIPES, pH 7.5, 0.1 mM EDTA, 0.015% Brij 35, 20% ethylene glycol, 2 mM dithiothreitol, 200 mM NaCl; Buffer F = 50 mM HEPES, pH 7.0, 0.1 mM EDTA, 0.015% Brij 35, 20% ethylene glycol, 0.2% β-mercaptoethanol; Buffer G = 50 mM PIPES, pH 7.5, 0.1 mM EDTA, 0.015% Brij 35, 0.2% β-mercaptoethanol, 20 mM NaCl, 100 mM sucrose; Buffer H = 50 mM PIPES, pH 7.5, 0.1 mM EDTA, 0.015% Brij 35, 40% ethylene glycol, 2 mM dithiothreitol, 100 mM KCl; bacterial media = 25 mM potassium phosphate, pH 7.5, containing (per liter) 4.37 g of NH_4Cl, 20 g of glucose, 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 50 mg of ampicillin, 232 mg of Mg^{2+}, 11 mg of Ca^{2+}, 10 mg of Fe^{2+}, 0.4 mg of Mn^{2+}, 2 mg of Zn^{2+}, 0.04 mg of Co^{2+}.

Expression of Abelson (ptab150) Kinase Activity in E. coli—Construction of the expression vector containing v-*abl* sequences has been described in detail elsewhere (21). In brief, sequences coding for the kinase domain of A-MuLV were transferred onto a vector which allows expression in *E. coli*. This vector, pCS4, contains the P_r promoter of λ bacteriophage, a ribosome-binding sequence, and an ATG codon, followed by 0.24 kilobase pairs of sequences coding for small t of simian virus 40 (22). In addition, a temperature-sensitive cI gene is present such that transcription from the P_r promoter is repressed at 30 °C and induced at 42 °C. To this vector, sequences coding for a specific N-terminal region of the A-MuLV kinase (from the *Hin*cII site at the *gag-abl* junction to the first *Pst*I site 1.2 kilobase pairs downstream) were placed in-frame behind the small t sequences. Thus at the permissive temperature, this plasmid expressed a fusion protein, termed the pt*abl*50 kinase, which consists of 80 amino acids of small t, 403 amino acids form the N-terminal domain of the viral Abelson kinase, and 5 amino acids at its C terminus from pBR322.

Bacteria were grown in a New Brunswick Scientific fermentor. Medium (9.5l) was inoculated with 100 ml of an overnight culture grown at 30 °C. Bacteria were grown for 7 h at 30 °C (until A_{660} = 4.0), followed by 4 h at 42.5 °C (final A_{660} = 13). Bacteria were harvested by centrifugation (12 min, 6500 × *g*), quick-frozen, and stored at −70 °C in 6 × 40-g aliquots.

Assay for ptabl50 Protein-Tyrosine Kinase Activity—Protein concentration was determined by the method of Bradford (23) using bovine serum albumin as a standard.

pt*abl*50 kinase activity was measured by the incorporation of ^{32}P from [γ-^{32}P]MgATP into angiotensin II. The standard assay consisted of 10 μl of pt*abl*50 kinase (in 0.1 mg/ml bovine serum albumin, 0.2% β-mercaptoethanol in Buffer A), 10 μl of angiotensin II (3 mg/ml in Buffer A) and was initiated by the addition of 10 μl of 15 mM Mg^{2+}, 0.3 mM [γ-^{32}P]ATP (1,500–10,000 cpm/pmol). After 30 min at 30 °C, the reaction was terminated by the addition of 120 μl of 10% (v/v) phosphoric acid. Each reaction tube was vortexed, and within 10 min, 120 μl of the reaction mixture was spotted onto phosphocellulose paper (2 cm square). Papers were washed extensively in 6% (v/v) acetic acid, washed once in acetone, dried, and counted. One unit of pt*abl*50 kinase activity is defined as 1 pmol of phosphate transferred from ATP to angiotensin II/min in the standard assay.

Purification of ptabl50 Kinase—A 40-g aliquot of bacteria was thawed on ice, washed once in 100 mM NaCl, 20 mM Tris-HCl, pH 8.8 (0 °C), 2 mM EDTA, and then lysed by sonication (4 × 30 s) in 300 ml of 40 mM NaCl, 1 mM EDTA, 100 mM sucrose, 0.015% Brij 35, 20 mM Tris-HCl, pH 8.8 (0 °C), 0.2% β-mercaptoethanol. Extracts were clarified by centrifugation (190,000 × *g*, 45 min), and the supernatants were applied batchwise to DE52 (220 g, wet weight) equilibrated in Buffer B. The DE52 was washed with Buffer 2, 20 mM NaCl, and pt*abl*50 kinase eluted by the application of Buffer B, 120 mM NaCl, 2 mM $MgCl_2$.

This preparation was applied batchwise to hydroxylapatite (400 g, wet weight, pre-equilibrated in Buffer C), the resin was then washed with Buffer C, 35 mM phosphate, and pt*abl*50 kinase eluted with Buffer C, 120 mM phosphate. The preparation was concentrated using Amicon stirred cells (YM-10 membranes) and dialyzed overnight against Buffer D. The next day, the material was applied to an Affi-Gel Blue column (50 ml bed volume) equilibrated in Buffer D, the column was washed with Buffer D, 220 mM NaCl, and the kinase eluted by the addition of Buffer D, 860 mM NaCl. The 10% ethylene glycol concentration in Buffer D allows the kinase activity to bind to the Affi-Gel Blue column. At 20% ethylene glycol, no binding was observed. Kinase activity was concentrated using an Amicon stirred cell (YM-10 membranes) and at the end of the second day, was applied to a Sephadex G-100 superfine column (2.6 × 95 cm, flow rate 8 ml h^{-1}) equilibrated in Buffer E. Fractions eluting from the column which contained kinase activity were pooled and dialyzed against Buffer F. The preparation was then applied to a Spherogel TSK DEAE-3SW high pressure liquid chromatography column (7.5 × 75 mm). The column was washed with Buffer F, 100 mM NaCl, and the kinase activity eluted with a 100–250 mM NaCl gradient (in Buffer F), 0.7 ml min^{-1} flow rate, with a total gradient volume of 120 ml.

Fractions containing kinase activity were pooled and dialyzed against Buffer G. The final purification step employed an anti-phosphotyrosine monoclonal antibody (MA-2G8) coupled covalently to Sepharose 4B (12). The preparation was applied to this column (2-ml bed volume), the column was washed with Buffer G, and kinase activity eluted by the addition of Buffer G, 2 mM phenyl phosphate (as a tyrosine phosphate analogue).

The final preparation was dialyzed against Buffer H and stored either at −20 °C, or quick-frozen and stored at −70 °C. The enzyme was stored in a dithiothreitol-containing buffer for long term stability.

RESULTS

Purification of ptabl50 Kinase—Sequences encoding the minimal kinase domain of A-MuLV were transferred into the vector pCS4, which allows expression in *E. coli*. This construct produces a fusion protein containing sequences derived from both small t protein of SV40 and the v-*abl* transforming gene which we have termed the pt*abl*50 kinase. The function of small t sequences in this construct was to increase the degree of expression in *E. coli* (21).

Purification of the pt*abl*50 kinase activity is summarized in Table I, based on a starting preparation of 40 g of *E. coli*. Preliminary experiments revealed the presence of at least five forms of the pt*abl*50 kinase which separated at various stages of the purification. Although all forms migrated reproducibly when rechromatographed, the ratio of the forms varied with different fermentations. Therefore, Table I represents averaged data obtained from multiple fermentations, with the data normalized with respect to the two forms which were purified (see below). These two forms constituted 35% of the total activity in the extract so that the actual specific activity of the extract was 206 ± 11 units mg^{-1} of protein (n = 10).[3] The various forms that were not purified were found in the following fractions: 3–25%[4] in the DE52-20 mM NaCl fraction, 22–48% in the hydroxylapatite-35 mM phosphate fraction, 6–60% in the Affi-Gel Blue-210 mM NaCl fraction, and 10% in the breakthrough of the anti-phosphotyrosine column.

On gel filtration, the enzyme moved as a monomer (M_r = 60,300 ± 700 (n = 3)). In the absence of NaCl in the column buffer, 25% of the enzyme moved with an apparent M_r = 125,000, and this form of the enzyme could be partially dissociated (up to 50%) to the M_r = 60,300 form if rechromatographed in the presence of 200 mM NaCl (data not shown).

The Abelson kinase is capable of an autophosphorylation reaction on tyrosine residues (17, 18, 24–26). We hoped to use this property as a purification step, by preincubating the enzyme with ATP followed by chromatography through an anti-phosphotyrosine monoclonal antibody column (12). Preliminary experiments revealed, however, that the two forms of pt*abl*50 kinase, as isolated in Step 5 and onward, were already phosphorylated, because over 80% of the activity was recovered in the fraction which bound specifically to the column even without preincubation with ATP. The 10% appearing in the breakthrough fraction of the column proba-

[3] Data presented in this manner represent the mean ± standard error, followed by the number of determinations (n) in parentheses.

[4] These figures represent the range of activities found in these fractions with various fermentations.

bly represented dephosphorylated forms of the enzyme, because on rechromatography, over 80% of this activity was again recovered in the breakthrough.

Criteria of Purity—The purified preparation (Step 7) was subjected to polyacrylamide gel electrophoresis (27), followed by silver staining (28) to determine the number of proteins present (Fig. 1). Using either low (35 ng) or high (425 ng) amounts of protein, two bands were apparent. At the high protein loading (Fig. 1, *lane 3*), one minor and two very faint bands of lower molecular weight were also observed. Whether these represent breakdown products or minor contaminants was not determined. The lower protein loading (Fig. 1, *lane 2*) showed that the slower migrating band represented the predominant species. The intensity of silver staining for 35 ng of pt*abl*50 kinase (as estimated by the method of Bradford (23)) is in reasonable agreement with the 40 ng loaded for each protein standard (compare Fig. 1, *lanes 1* and *2*).

In comparison to proteins of known molecular weight (Fig. 1, *lane 1*), the two major bands have a M_r = 59,800 and 57,200. Based on an apparent M_r = 60,300 on gel filtration, the enzyme appears to be a monomer. These estimates of the molecular weight are in good agreement with a theoretical M_r = 56,000 based on the known DNA sequence.

To examine the immunological parameters of the enzyme, portions of the preparation from Step 7 were incubated with 5 mM Mg^{2+}, 100 μM [γ-^{32}P]ATP for 30 min at 30 °C. One reaction was terminated by the addition of 15 mM EDTA, while two others were immunoprecipitated by the addition of a monoclonal antibody directed against small t protein of SV40 (antibody 419, kindly provided by Dr. Ed Harlow, Cold Spring Harbor Laboratory) or with a polyclonal serum against v-*abl*-specific sequences (29). All three samples were subjected

TABLE I
Purification of ptabl50 kinase

Step	Volume	Units	Protein	Specific activity	Yield	Purification
	ml	*pmol min*$^{-1}$	*mg*	*units mg*$^{-1}$	%	*-fold*
1. Extract	300	101,990	1,412	72	100	1
2. DE52	3,000	95,870	393	244	94	3.4
3. Hydroxylapatite	3,000	90,120	108	834	88	11.6
4. Affi-Gel Blue	850	75,600	33	2,320	75	32
5. Sephadex G-100 superfine	17	54,050	7	7,720	53	107
6. TSK DEAE-3SW	10	37,840	1.37	27,620	37	384
7. Anti-phosphotyrosine	6	31,620	0.117	270,000	31	3,750

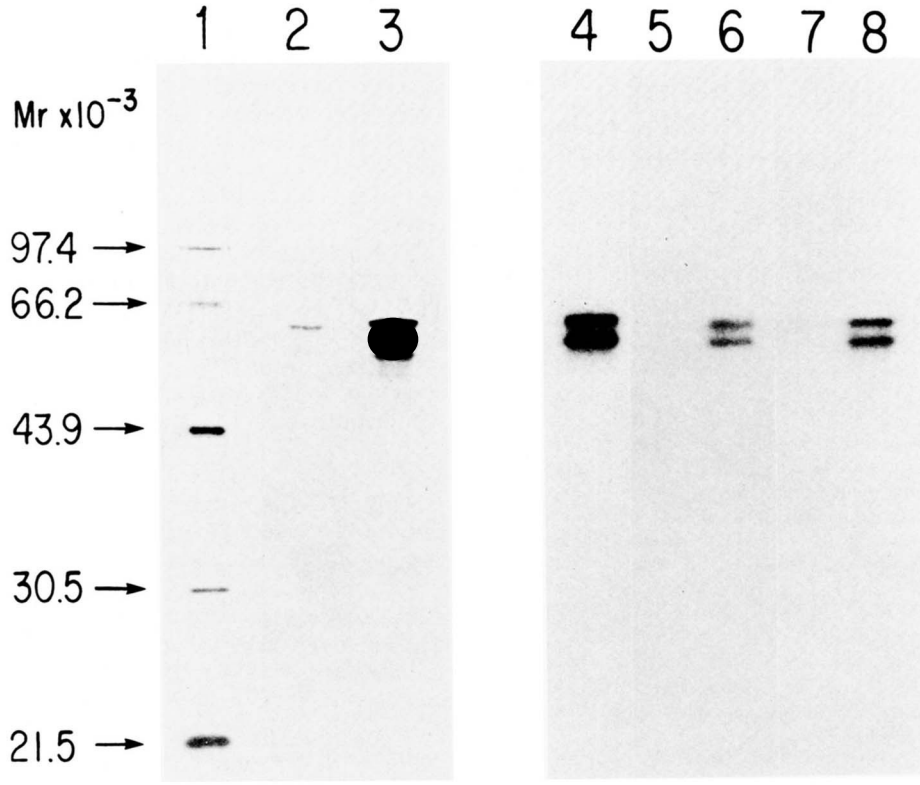

FIG. 1. **SDS-polyacrylamide gel electrophoresis of purified pt*abl*50 kinase.** Portions of the purified enzyme (from Step 7) were subjected to SDS-polyacrylamide gel electrophoresis (10% gel), followed by silver staining. *Lane 1* represents protein markers of known molecular weight, with 40 ng of each marker loaded. *Lane 2*, 35 ng and *lane 3*, 425 ng of the purified pt*abl*50 kinase. *Lanes 4–8* represent an autoradiograph of the pt*abl*50 kinase, incubated with [γ-^{32}P]MgATP, and subjected to SDS-polyacrylamide gel electrophoresis. *Lane 4*, pt*abl*50 kinase incubated with MgATP. *Lanes 5–8*, pt*abl*50 kinase ^{32}P-labeled and immunoprecipitated with either preimmune serum (*lane 5*) followed by immune Abelson-specific serum (*lane 6*), or a nonspecific monoclonal antibody (*lane 7*) followed by specific small t monoclonal antibody (*lane 8*). Immunoprecipitates were collected with *Staphylococcus aureus* and washed in 1 mM EDTA, 1% deoxycholate, 0.1% SDS, 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, prior to dissociation with sample buffer. Further details are provided in the text.

to SDS-polyacrylamide gel electrophoresis. In the sample treated with [γ-^{32}P]ATP alone, two phosphorylated bands were evident (Fig. 1, *lane 4*) which co-migrated with the two major protein bands visualized by silver staining (Fig. 1, *lanes 2* and *3*). Phosphoamino acid analysis (30) of these two bands revealed phosphotyrosine as the exclusive amino acid phosphate acceptor (data not shown). Immunoprecipitation with a nonspecific monoclonal antibody (Fig. 1, *lane 7*) or preimmune mouse serum (Fig. 1, *lane 5*) demonstrated the absence of nonspecific binding, while both phosphorylated bands were immunoprecipitated by the addition of either v-*abl*-specific serum (Fig. 1, *lane 6*) or the anti-small t monoclonal antibody (Fig. 1, *lane 8*). Thus, the purified enzyme is a small t-v-*abl* fusion protein as expected from the sequences in the plasmid expressed in *E. coli*.

The purification scheme, as summarized in Table I, allowed 117 μg of essentially homogeneous pt*abl*50 kinase to be obtained from 40 g of *E. coli* within 6–7 days. Stored at −20 °C in Buffer H, the enzyme lost less than 20% of its activity over 4 months. Enzyme activity also survived quick-freezing and storage at −70 °C, but the long term stability under these conditions was not determined.

Kinetic Properties—All of the following reactions were carried out using the Step 7 purified pt*abl*50 kinase. In a standard assay with angiotensin (1 mg ml^{-1}) as the substrate, the kinase activity was stable, and phosphate incorporation was linear for at least 45 min. In addition, incorporation was linear up to at least 40 pmol of phosphate. The pH optimum for this reaction was pH 7.5, with greater than 50% of the maximal activity expressed over the range pH 6.5–8.5.

A number of protein-tyrosine kinases, including the Abelson kinase, show an apparent preference for Mn^{2+} ions over Mg^{2+}, at least when their kinase activity is measured using partially purified preparations (1, 4, 17, 32, 33). It has been suggested that this may be due to the ability of Mn^{2+} ions, but not Mg^{2+} ions, to inhibit protein-tyrosine phosphatases, present as contaminating activities in crude systems (34, 35). With purified pt*abl*50 kinase, Mn^{2+} ions were found to be only partially effective (Fig. 2), with less than 20% of the activity expressed compared to that observed at saturating concentrations of Mg^{2+} ions. Half-maximal activity was observed at 1.5 mM Mg^{2+} ions.

The inclusion of either K^+ or Na^+ ions (up to 250 mM) in the standard assay had no effect. Increasing ionic strength has been reported to inhibit other protein-tyrosine kinases (15, 36) but the degree of inhibition is dependent on the substrate employed (37). In the absence of Mg^{2+}, Ca^{2+} ions (over the range 0.1–5.0 mM) failed to support enzyme activity. In the standard assay, the addition of either Ca^{2+}-calmodulin (10–200 μM Ca^{2+} and up to 1 μM calmodulin), cAMP (0.1–100 μM), or the specific heat-stable protein inhibitor of the cAMP-dependent protein kinase (at levels which produce greater than 95% inhibition of the cAMP-dependent kinase) had no effect on the activity of the pt*abl*50 kinase.

We have also determined the kinetic parameters for a number of substrates under our standard assay conditions (Table II). For these experiments, preliminary values of the K_m were estimated from two substrate concentrations using the Eisenthal-Cornish-Bowden plot (38). We then determined K_m and V using a series of substrate concentrations, spanning up to a 10-fold range around the estimated K_m. For all substrates, linearity with respect to both time and enzyme concentration was established. Each data point was performed in triplicate. Data were then analyzed by a least-squares fit to a hyperbola of V *versus* $[S]$ measurements (39, 40), and by the computerized direct linear method (38, 41). The results from both methods were weight-averaged to give the data in Table II.

For angiotensin II, the K_m was 3.7 ± 0.3 mM (Table II). The K_m for ATP was found to be dependent on the concentration of angiotensin II, measured as 39 μM at 0.95 mM angiotensin and 100 μM at 11.47 mM angiotensin II. From the turnover rates for ATP at these two concentrations of angiotensin II, one can calculate the K_m for angiotensin II, at saturating concentrations of ATP, to be 15.8 mM. The dependence of the apparent K_m for one substrate on the concentration of the second suggests that the binding sites for the two substrates overlap.

The turnover number of 170 μmol min^{-1} μmol^{-1} of pt*abl*50 kinase, determined for ATP at high levels of angiotensin II (Table II), is comparable to the specific activity of the most active protein-serine kinases (42–44). To date, the highest turnover numbers reported for other protein-tyrosine kinases are (calculation based on a 30 °C incubation) 15–30 μmol min^{-1} μmol^{-1} for the insulin receptor (8, 45, 46), 7–55 μmol min^{-1} μmol^{-1} for the EGF receptor (45, 47), and 2–3 μmol min^{-1} μmol^{-1} for the transforming protein of Rous sarcoma virus pp60^{src}, (15, 16).

pt*abl*50 kinase can use GTP as the phosphate donor (Table

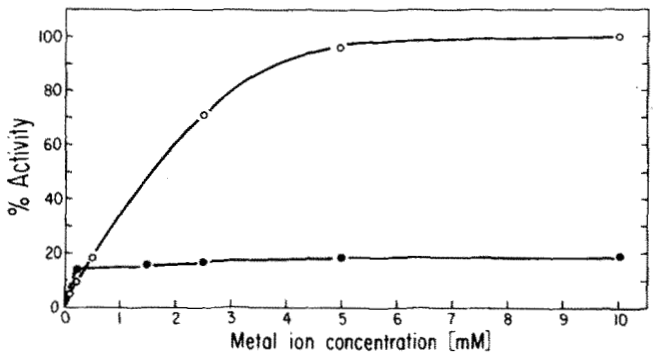

FIG. 2. **The effect of Mg^{2+} and Mn^{2+} ions on the activity of the pt*abl*50 kinase.** Incubations were carried out under standard assay conditions (1 mg ml^{-1} of angiotensin II, 100 μM ATP, 30 min, 30 °C, 10 units ml^{-1} of pt*abl*50 kinase) in the presence of either Mg^{2+} ions (○) or Mn^{2+} ions (●). Activity is expressed as a percentage of the maximum kinase activity observed at saturating concentrations of Mg^{2+} ions (5 mM $MgCl_2$). No kinase activity was observed in the absence of Mg^{2+} ions.

TABLE II

Kinetic constants for substrates of ptabl50 kinase

Substrate	K_m	Turnover number
	mM	*μmol min^{-1} mg^{-1} kinase*
1. Angiotensin II (at 100 μM ATP)	3.7 ± 0.3[a]	1.25 ± 0.05
2. ATP (at 0.956 mM angiotensin II)	0.039 ± 0.001	0.38 ± 0.02
3. ATP (at 11.47 mM angiotensin II)	0.100 ± 0.003	2.82 ± 0.03
4. GTP (at 0.956 mM angiotensin II)	0.3 ± 0.04	0.19 ± 0.02
5. Lys-Tyr-Lys (at 100 μM ATP)	12 ± 1.2	0.11 ± 0.004
6. Arg-Tyr (at 100 μM ATP)	36 ± 5	0.1 ± 0.007
7. Tyr (at 100 μM ATP)	ND[b]	~0.00026[c]

[a] Values represent mean ± S.E.
[b] ND, not determined.
[c] Determined at 0.2 mg ml^{-1} of tyrosine.

II), but the K_m is nearly 8-fold higher and the turnover number 2-fold lower than the corresponding values determined for ATP. GTP supports the activity of pp60^{src} (16) and possibly the platelet-derived growth factor receptor (4). In contrast, the protein-tyrosine kinase activities associated with the transforming protein of Snyder-Theilen feline sarcoma virus (48), the insulin receptor (33, 49), as well as a tyrosine kinase isolated from normal rat liver (37), all appear to have a strict requirement for ATP. The situation with the EGF receptor remains to be clarified (45, 50–52).

Because angiotensin II, an octapeptide, appeared to be a good phosphate acceptor, we were interested to determine the minimum size of peptide which would serve as a substrate. Table II shows that peptides containing either three or two amino acids or even simply free tyrosine could also be phosphorylated, although the latter substrate is particularly ineffective.

Specificity of ptabl50 Kinase for Tyrosine Residues—Incubation of the pt*abl*50 kinase with a variety of protein substrates and a number of different peptides, followed by phosphoamino acid analysis, revealed that phosphotyrosine was the exclusive amino acid phosphate acceptor (data not shown). Incubation of the peptide Lys-Ser-Tyr with a high concentration of the pt*abl*50 kinase, followed by phosphoamino acid analysis, revealed trace amounts of phosphoserine (Fig. 3, *A* and *B*), but such low levels could be derived nonenzymatically from the adjacent phosphotyrosine during preparation. To examine critically whether serine residues can be substrates for the pt*abl*50 kinase, we used the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly, which does not contain tyrosine residues. Incorporation of ^{32}P suggested a very low rate of phosphorylation, approximately 10^{-4} of the rate observed with angiotensin II. Surprisingly, however, phosphoamino acid analysis revealed only phosphotyrosine (Fig. 3*C*), indicating that at least one of the amino acids used to synthesize the commercially obtained peptide was contaminated with traces of tyrosine. An analogue of this peptide, which contained tyrosine at position 5 instead of serine, has been reported to be phosphorylated by both the EGF (53) and insulin (11) receptors.

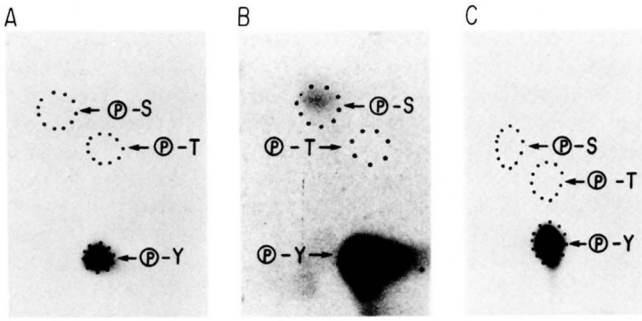

FIG. 3. **Phosphoamino acid analysis of substrates for pt*abl*50 kinase.** *A* and *B*, 12.5 units of pt*abl*50 kinase was incubated with 25 μg of Lys-Ser-Tyr and 100 μM [γ-^{32}P]ATP (20,000 cpm pmol^{-1}) in 30 μl for 60 min at 30 °C. The phosphorylated peptide was purified by paper electrophoresis (pH 3.5, 2,400 V, 90 min), eluted, and subjected to acid hydrolysis (6 N HCl, 105 °C, 60 min). Phosphoamino acids were separated by two-dimensional thin layer electrophoresis (12, 30), internal standard markers were stained with ninhydrin, and ^{32}P-labeled phosphoamino acids were detected by autoradiography. *A* represents a 10-h exposure; *B*, a 1-month exposure. Control incubations containing only peptide and ATP and processed as above revealed only traces of ^{32}P associated with serine and tyrosine, at a level barely detectable above background (data not shown). *C*, 60 units of pt*abl*50 kinase was incubated with 88.5 μg of the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly and 100 μM ATP (15,000 cpm pmol^{-1}) in 30 μl for 5 h at 30 °C. The reaction was terminated by the addition of 100 μl of 10% (v/v) phosphoric acid, and the peptide was isolated by spotting the reaction mixture on phosphocellulose paper and was separated from ATP by washing the paper in acetic acid (6%). The peptide was eluted by the addition of ammonium hydroxide and lyophilized, and the phosphoamino acid analysis was performed as described above.

Autophosphorylation of ptabl50 Kinase—Most protein kinases undergo an autophosphorylation reaction when incubated with MgATP (1, 54). Previous work had demonstrated that the Abelson kinase can undergo intermolecular autophosphorylation both *in vivo* and *in vitro* when immunoprecipitated from virally transformed cells (25, 26). Earlier in this paper, we showed that homogeneous pt*abl*50 kinase, as isolated from *E. coli*, could also be autophosphorylated (Fig. 1), suggesting that this reaction is not due to a trace contaminating *E. coli* protein-tyrosine kinase. The purified kinase incorporates about 1.0 mol of phosphate/mol of enzyme (Fig. 4). The autophosphorylation of pp60^{src} (16, 55), the EGF receptor (56), and the insulin receptor (32) has also been reported to result in the incorporation of significant amounts of phosphate. In the case of pp60^{src} (57) and the insulin receptor (49, 58), autophosphorylation has been correlated with activation. An increased phosphotyrosine content of the protein-tyrosine kinase of PRC II virus is also associated with an increase in kinase activity (59). The normal v-*abl* protein contains two major sites of tyrosine phosphorylation (26, 60–62), but the stoichiometry of phosphorylation has not been reported. At an enzyme concentration of 43.6 nM, the rate of phosphorylation at 30 °C was approximately 1.3 nmol of phosphate min^{-1} mg^{-1} of kinase. This figure is almost identical to the rate reported for pp60^{src} autophosphorylation (16).

In attempting to examine the phosphorylation of other proteins of a similar molecular weight to the pt*abl*50 kinase, we had thought to mask the autophosphorylation reaction by incubating the kinase with unlabeled MgATP prior to the addition of exogenous substrate and [^{32}P]ATP. This failed to have any effect on the autophosphorylation reaction. To examine this failure, we incubated two identical reactions, each containing the pt*abl*50 kinase and MgATP, and to one

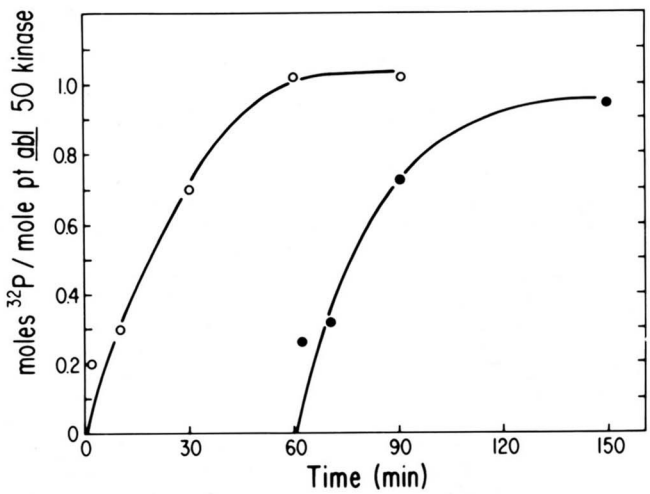

FIG. 4. **Autophosphorylation of pt*abl*50 kinase.** Two tubes containing 0.654 pmol of pt*abl*50 kinase were incubated for various times with 0.37 mM ATP, 5 mM Mg^{2+} (15-μl reaction, 30 °C). To one tube (○), [γ-^{32}P]ATP was added at zero time (final specific activity 38,000 cpm pmol^{-1}). To the other tube (●) [γ-^{32}P]ATP was added after 1 h. Reactions were terminated by the addition of 2 μl of 250 mM EDTA, and products were then subjected to SDS-polyacrylamide gel electrophoresis (10% gels). The pt*abl*50 kinase was located by autoradiography, and the bands were excised and counted.

tube we added trace concentrations of high specific activity [γ-^{32}P]MgATP at zero time. By ^{32}P incorporation, the autophosphorylation reaction appeared to have terminated after 1 h (Fig. 4). Addition of [γ-^{32}P]MgATP to an identical reaction incubated for 1 h with MgATP but without radiolabel, however, resulted in ^{32}P incorporation into pt*abl*50 kinase at the same rate and to the same extent (Fig. 4). It therefore appeared that a ^{32}P exchange reaction was occurring in the pt*abl*50 kinase.

To further analyze this phenomenon, we incubated pt*abl*50 kinase with [γ-^{32}P]MgATP, filtered the preparation to remove ATP, and then added EDTA, unlabeled ADP, or unlabeled ATP (Fig. 5). Control incubations with EDTA indicated no formation of $^{32}P_i$ (Fig. 5*A*) and therefore, the absence of phosphotyrosyl-protein phosphatase activities which show optimal activity in the presence of EDTA (34). Addition of MgADP resulted in the dephosphorylation of the pt*abl*50 kinase (Fig. 5*A*) and the synthesis of [^{32}P]ATP (Fig. 5*B*). Similarly, addition of unlabeled ATP to ^{32}P-labeled pt*abl*50 kinase resulted in the loss of ^{32}P from the protein and the synthesis of [^{32}P]ATP (Fig. 5*B*, *lane 11*). The increased amount of $^{32}P_i$ generated in this experiment (Fig. 5*B*, *lane 11*) demonstrated that the pt*abl*50 kinase preparation contained a very low level of ATPase activity (<0.1 nmol min^{-1} mg^{-1} of kinase). Although $^{32}P_i$ was also observed in the experiments with ADP, this was present at zero time and did not increase during incubation. The absence of detectable ATPase presumably reflects the low concentrations of ATP formed in these experiments carried out with ADP. The cAMP-dependent protein kinase has also been shown to possess a low level of ATPase activity (63).

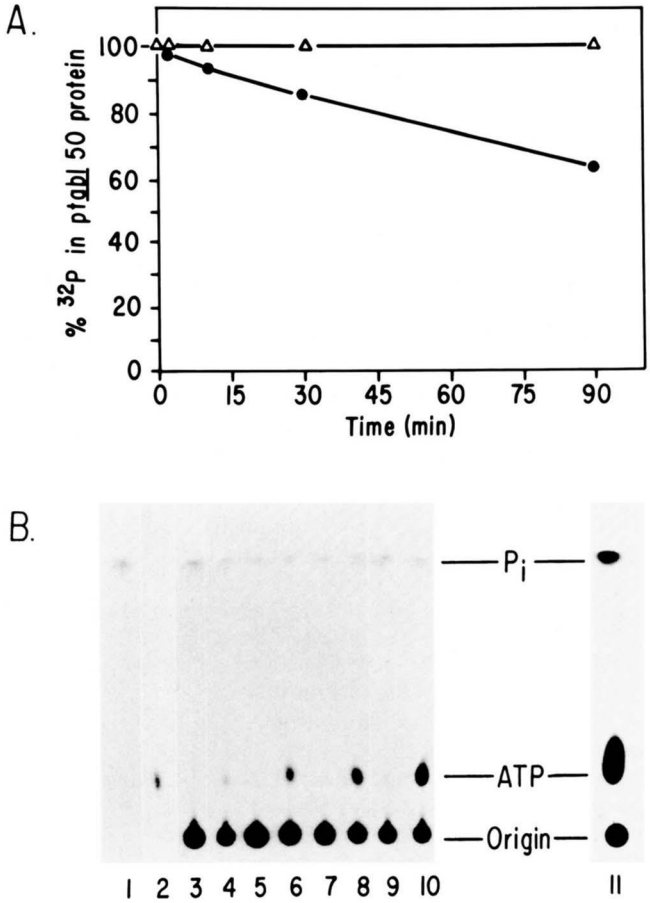

FIG. 5. **Reversibility of the pt*abl*50 kinase autophosphorylation reaction.** pt*abl*50 kinase was incubated overnight at 4 °C with [γ^{32}P]ATP (30,000 cpm pmol^{-1}), followed by gel filtration (Sephadex G-50 superfine) to remove the ATP. Portions containing 0.24 pmol of ^{32}P-labeled pt*abl*50 kinase were incubated with 10 mM Mg^{2+}, 1 mM ADP, 5 mM Mg^{2+}, 0.37 mM ATP, or 1 mM EDTA at 30 °C for various times as indicated. The reaction volume was 15 μl. The reaction was terminated by the addition of 2 μl of 200 mM EDTA. *A*, per cent phosphorylation of the protein following incubation with ADP (●) or EDTA (△) was determined by subjecting 14 μl of the reaction to SDS-polyacrylamide gel electrophoresis, locating the ^{32}P-labeled kinase by autoradiography, excising the ^{32}P-labeled bands, and counting. *B*, portions of 1 μl of the reaction mixture were spotted onto polyethyleneimine thin layer-cellulose (Macherey-Nagel, Düren, West Germany), chromatographed with 0.5 M KH_2PO_4, pH 3.5, as the solvent, and [^{32}P]ATP formation was analyzed by autoradiography. *Lane 1*, $^{32}P_i$ standard; *lane 2*, [γ-^{32}P]ATP standard; *lanes 3, 5, 7,* and *9*, ^{32}P-pt*abl*50 kinase incubated with EDTA for 2, 10, 30, and 90 min, respectively; *lanes 4, 6, 8,* and *10*, ^{32}P-pt*abl*50 kinase incubated with ADP for 2, 10, 30, and 90 min, respectively; *lane 11*, $^{32}P_i$-labeled pt*abl*50 kinase incubated with ATP (unlabeled, 0.37 mM) for 30 min and analyzed for [^{32}P]ATP formation as described above.

DISCUSSION

We report here the first purification of the protein-tyrosine kinase activity encoded by the transforming gene of Abelson murine leukemia virus. This is also the first active protein-tyrosine kinase to be purified using an expression vector system in *E. coli.* The transforming protein of Rous sarcoma virus has been expressed in *E. coli* and large quantities of the protein were produced, but the majority of the material proved to be insoluble (64, 65). In contrast, in our experiments, only low concentrations of the Abelson pt*abl*50 kinase were synthesized, but the material was in an active, soluble form. Purification proved to be difficult because multiple forms were found to exist; at least six species could be separated chromatographically. The reason for this is unclear, but some of the forms are probably generated as a consequence of multiple potential initiation sites in the vector. The different forms could also result from variable degrees of phosphorylation or proteolytic breakdown products formed during fermentation. Immunoprecipitation of extracts from radiolabeled bacteria (^{32}P or ^{35}S), with antibodies directed against the Abelson kinase, also revealed multiple species of pt*abl*50 kinase (21). With respect to the two forms which were purified, the overall purification was 3750-fold with a 31% yield, such that 117 μg of pt*abl*50 kinase was obtained from 40 g of *E. coli* within 6–7 days. Assuming all forms have equal activity, the total pt*abl*50 kinase in *E. coli* extracts would represent only 0.076% of the soluble protein. The most powerful purification step was the anti-phosphotyrosine monoclonal antibody column, where a 10-fold purification to homogeneity was obtained. This column also binds the platelet-derived growth factor receptor, the EGF receptor, and the p120$^{gag\text{-}abl}$ protein of A-MuLV (5, 12). This step could not be used earlier in our protocol because expression of the Abelson kinase in *E. coli* results in the phosphorylation of many bacterial proteins on tyrosine (18). A 10–15-fold purification was also possible on the ion-exchange high pressure liquid chromatography column, but only by accepting a significant reduction in yield (data not shown).

The final preparation contained two major proteins as visualized by silver staining following SDS-polyacrylamide gel electrophoresis. Both of these proteins were immunoprecipitated by antibodies directed against either the small t antigen region or the Abelson kinase domain, providing strong evidence for their identity as species of pt*abl*50 kinase. Although both protein bands become phosphorylated in the presence of MgATP, it remains to be shown that both bands possess kinase activity. An earlier purification protocol we

had developed used a narrow range of salt concentrations to elute stepwise the kinase from the Affi-Gel Blue column. This procedure resulted in a single species of pt*abl*50 kinase (data not shown), suggesting that the two forms can be separated by this step. We have yet to determine the exact relationship between these two species. Autophosphorylation of the protein-tyrosine kinases associated with either PRC II (59) or Rous sarcoma virus (55) results in the appearance of two forms on SDS-polyacrylamide gel electrophoresis. The most highly phosphorylated form of each protein has the slowest mobility.

The use of angiotensin II as a phosphate acceptor was based on the procedure developed by Wong and Goldberg (66, 67). This is a particularly powerful assay as it allows the measurement of a protein-tyrosine kinase even in cell extracts, because background phosphorylation due to endogenous kinases and their substrates can be removed by precipitation with trichloroacetic acid under conditions where angiotensin II is soluble. In *E. coli* extracts, background phosphorylation is so low that the pt*abl*50 kinase can be measured accurately even without the protein precipitation step.

The high K_m for angiotensin II is not unusual for peptides when they are used as substrates for protein-tyrosine kinases (11, 44, 50, 53, 67, 68). As suggested by Pike *et al.* (53), this may reflect either incorrect primary sequence determinants or the small size of the substrate. Factors other than the primary sequence are important for the cAMP-dependent kinase (69, 70). There are some data to suggest that acidic residues in the vicinity of and a basic amino acid 6–7 residues N-terminal to the phosphorylated tyrosine may be important recognition determinants for protein-tyrosine kinases. There are also, however, clear exceptions to this rule (13, 62), particularly as applied to the Abelson kinase (62, 71). In this paper, we have shown that peptides containing just three or even two amino acids could be phosphorylated, as well as free tyrosine, although this latter substrate is particularly ineffective. Both Tyr-Arg and free tyrosine have been tested as substrates for a number of protein-tyrosine kinases and were not phosphorylated (50, 67, 68, 72). Tyr-Arg is phosphorylated by pt*abl*50 kinase but high concentrations are inhibitory (data not shown). Limited phosphorylation of Tyr-Arg has also been reported for the insulin receptor (73). Tyramine and *N*-acetyltyrosine are phosphorylated by immunoprecipitates containing the $p130^{gag\text{-}fps}$ protein of Fujinami sarcoma virus (72).

For all the substrates of pt*abl*50 kinase, tyrosine was the exclusive amino acid phosphate acceptor. With Lys-Ser-Tyr, a very small amount of serine phosphorylation was also observed, but we consider it unlikely that this was derived enzymatically.

Perhaps all protein kinases, and certainly the Abelson kinase, can carry out an autophosphorylation reaction (1, 54). The pt*abl*50 kinase must also undergo autophosphorylation when expressed in *E. coli*, because most of the activity binds to the anti-phosphotyrosine monoclonal column. The autophosphorylation of pt*abl*50 kinase observed here *in vitro* (Fig. 4), however, appears not to be a true phosphorylation, but may represent an exchange reaction between the unlabeled phosphate present in the pt*abl*50 kinase with ^{32}P from [γ-^{32}P]ATP. The rate of autophosphorylation would therefore represent the rate of exchange and the plateau at 1.0 mol of phosphate, the point at which the ^{32}P-specific activity of the enzyme would be equal to the specific activity of the [γ-^{32}P]ATP. The purified pt*abl*50 kinase used in these experiments is a monomer, which binds to the anti-phosphotyrosine monoclonal column, indicating that each molecule of kinase already contained at least one molecule of phosphotyrosine prior to incubation with MgATP *in vitro*. The autophosphorylation reactions of several protein-serine kinases have also been shown to be reversible (54, 74, 75), and there is at least one example of a phosphotyrosyl-protein where the free energy of hydrolysis of phosphotyrosine was shown to be only slightly lower than that of the terminal phosphate residue in ATP (76). The autophosphorylation of pt*abl*50 kinase could represent an equilibrium phenomenon, driven at zero time by the enzyme being fully phosphorylated, using low levels of ADP (present as a contaminant in ATP or generated by the low ATPase activity) to first dephosphorylate the enzyme, and thereby allow subsequent rephosphorylation by [γ-^{32}P]ATP. Alternatively, the enzyme may simply bind ATP and directly exchange the γ-phosphate of ATP with the phosphate already present on the enzyme. Presently, we are performing a detailed analysis to determine the exact mechanism involved.

Recently, two other activities have been found in association with protein-tyrosine kinases. First, the EGF receptor has been reported to act as an ATP-stimulated nuclease (77). pt*abl*50 kinase is completely devoid of such an activity.[5] Second, the transforming proteins $pp60^{src}$ (78) and $pp68^{ros}$ (79) have been found in association with a phosphatidylinositol kinase activity. Again, the pt*abl*50 kinase lacks such an activity.[6] The specific activity of purified pt*abl*50 kinase is over 170 μmol of phosphate min^{-1} μmol^{-1}, strongly suggesting that the ability of this protein to phosphorylate tyrosine residues is not a trace enzymatic activity.

Finally, we have tested cAMP, the cAMP-dependent protein kinase inhibitor protein, Ca^{2+}, and Ca^{2+}-calmodulin as possible regulators of the pt*abl*50 kinase, all without effect. The Abelson kinase we have purified however, represents only the catalytic domain (20); it may lack regulatory sequences. In this respect, pt*abl*50 kinase would be analogous to the trypsin-derived catalytic fragments of the cAMP- and cGMP-dependent protein kinases and protein kinase C, which retain activity but no longer respond to their normal physiological modulators (80). Protease-resistant but enzymatically active domains of $pp60^{src}$ (81, 82) and $p90^{yes}$ (81) have also been reported. In addition, the N terminus of pt*abl*50 kinase contains sequences derived from small t of SV40, while the normal viral enzyme contains *gag* sequences derived from Moloney murine leukemia virus (19, 20) which have a myristate group at their N terminus (83, 84). One might speculate that the modified N terminus of pt*abl*50 kinase could result in an alteration of substrate specificity, for instance, the absence of phosphatidylinositol kinase activity. For both protein kinase C (85) and $pp60^{src}$ (15, 16), evidence exists which suggests that alterations of specificity may occur when the size of the catalytic domain is altered. By microinjection of pt*abl*50 kinase into cells, however, we have demonstrated that the enzyme retains at least some of the biological activities associated with the protein-tyrosine kinase encoded by the normal v-*abl* gene, namely, the regulation of ribosomal protein S6 phosphorylation on serine residues (86) and the ability to induce morphological transformation of NIH/3T3 fibroblasts.[7]

Acknowledgments—We would like to thank Sy-dar Wang and Daniel Wang (Massachusetts Institute of Technology, Cambridge, MA) for use of their fermentor and their help in growing the bacteria

[5] B. Mroczkowski, S. Cohen, and J. G. Foulkes, unpublished observations.

[6] M. Whitman, L. Cantley, and J. G. Foulkes, unpublished observations.

[7] B. Shephard, L. B. Chen, J. G. Foulkes, and D. Baltimore, unpublished observations.

used in these studies. We would also like to acknowledge Jean Wang (University of California, San Diego, CA) for providing the *E. coli* containing the pt*abl*50 kinase plasmid, and Tom Soderling for phosphorylase kinase and Jackie Corbin (Howard Hughes Institute, Nashville, TN) for the catalytic subunit of the cAMP-dependent protein kinase. Finally, we would like to thank John Port (Massachusetts Institute of Technology, Cambridge, MA) for his help in the analysis of the enzyme kinetic data.

REFERENCES

1. Hunter, T., and Sefton, B. (1981) in *Molecular Aspects of Cellular Regulation* (Cohen, P., and van Heyningan, S., eds) Vol. 2, pp. 337–370, Elsevier/North-Holland, New York
2. Bishop, M. (1983) *Annu. Rev. Biochem.* **52,** 302–354, New York
3. Ushiro, H., and Cohen, S. (1980) *J. Biol. Chem.* **255,** 8363–8365
4. Ek, B., and Heldin, C.-H. (1982) *J. Biol. Chem.* **257,** 10486–10492
5. Frackelton, A. R., Jr., Tremble, P. M., and Williams, L. T. (1984) *J. Biol. Chem.* **259,** 7909–7915
6. Rubin, J. B., Shia, M. A., and Pilch, P. F. (1983) *Nature* **305,** 438–440
7. Jacobs, S., Kull, F. C., Jr., Earp, H. S., Svoboda, M. E., Van Wyk, J. J., and Cuatrecasas, P. (1983) *J. Biol. Chem.* **258,** 9581–9584
8. Nemenoff, R. A., Kwok, Y. C., Shulman, G. I., Blackshear, P. J., Osathanondh, R., and Avruch, J. (1984) *J. Biol. Chem.* **259,** 5058–5065
9. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., White, M., and Khan, C. R. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 2137–2141
10. Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M., and Kahn, R. C. (1982) *Nature* **298,** 667–669
11. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., White, M. F., and Kahn, C. R. (1983) *J. Biol. Chem.* **258,** 10973–10980
12. Frackelton, A. R., Jr., Ross, A. H., and Eisen, H. N. (1983) *Mol. Cell. Biol.* **3,** 1343–1352
13. Cooper, J. A., Esch, F. S., Taylor, S. S., and Hunter, T. (1984) *J. Biol. Chem.* **259,** 7835–7841
14. Cooper, J. A., and Hunter, T. (1983) *Curr. Top. Microbiol. Immunol.* **107,** 125–161
15. Richert, N. D., Blithe, D. L., and Pastan, I. (1982) *J. Biol. Chem.* **257,** 7143–7150
16. Graziani, Y., Erikson, E., and Erikson, R. L. (1983) *J. Biol. Chem.* **258,** 6344–6351
17. Witte, O., Dasgupta, A., and Baltimore, D. (1980) *Nature* **283,** 826–831
18. Wang, J. Y. J., Queen, C., and Baltimore, D. (1982) *J. Biol. Chem.* **257,** 13181–13184
19. Goff, S., and Baltimore, D. (1982) *Adv. Viral. Oncol.* **1,** 127–139
20. Prywes, R., Foulkes, J. G., Rosenberg, N., and Baltimore, D. (1983) *Cell* **34,** 569–579
21. Wang, J. Y. J., and Baltimore, D. (1985) *J. Biol. Chem.* **260,** 64–71
22. Queen, C. (1983) *J. Mol. Appl. Genet.* **2,** 1–10
23. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
24. Watanabe, S. M., Rosenberg, N. E., and Witte, O. N. (1984) *J. Virol.* **51,** 620–627
25. Witte, O. N., Goff, S., Rosenberg, N., and Baltimore, D. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 4993–4997
26. Ponticelli, A. S., Whitlock, C. A., Rosenberg, N., and Witte, O. N. (1982) *Cell* **29,** 953–960
27. Laemmli, U. K. (1970) *Nature* **227,** 680–685
28. Merril, C. R., Goldman, D., Sedman, S. A., and Ebert, M. H. (1980) *Science* **211,** 1437–1438
29. Witte, O. N., Rosenberg, N., and Baltimore, D. (1979) *J. Virol.* **31,** 776–784
30. Hunter, T., and Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 1311–1315
31. Feldman, R. A., Wang, L. H., Hanafusa, H., and Balduzzi, P. C. (1982) *J. Virol.* **42,** 228–236
32. White, M. F., Haring, H. U., Kasuga, M., and Kahn, C. R. (1984) *J. Biol. Chem.* **259,** 255–264
33. Zick, Y., Kasuga, M., Kahn, C. R., and Roth, J. (1983) *J. Biol. Chem.* **258,** 75–80
34. Foulkes, J. G. (1983) *Curr. Top. Microbiol. Immunol.* **107,** 163–180
35. Casnellie, J. E., Harrison, M. L., Pike, L. J., Hellstrom, K. E., and Krebs, E. G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 282–286
36. Tuy, F. P. D., Henry, J., Rosenfeld, C., and Kahn, A. (1983) *Nature* **305,** 435–438
37. Wong, T. W., and Goldberg, A. R. (1984) *J. Biol. Chem.* **259,** 8505–8512
38. Eisenthal, R., and Cornish-Bowden, A. (1974) *Biochem. J.* **139,** 715–720
39. Cleland, W. W. (1967) *Adv. Enzymol.* **29,** 1–32
40. Wilkinson, G. N. (1961) *Biochem. J.* **80,** 324–332
41. Cornish-Bowden, A., and Trager, W. F. (1978) *J. Theor. Biol.* **74,** 163–175
42. Sugden, P. H., Holladay, L. A., Reimann, E. M., and Corbin, J. D. (1976) *Biochem. J.* **159,** 409–422
43. Bechtel, P. J., Beavo, J. A., and Krebs, E. G. (1977) *J. Biol. Chem.* **252,** 2691–2697
44. Tessmer, G. W., Skuster, J. R., Tabatabai, L. B., and Graves, D. J. (1977) *J. Biol. Chem.* **252,** 5666–5671
45. Pike, L., Kuenzel, E. A., Casnellie, J. E., and Krebs, E. G. (1984) *J. Biol. Chem.* **259,** 9913–9921
46. Petruzzelli, L., Herrera, R., and Rosen, O. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 3327–3331
47. Parker, P. J., Young, S., Gullick, W. J., Mayes, E. L. V., Bennett, P., and Waterfield, M. D. (1984) *J. Biol. Chem.* **259,** 9906–9912
48. Snyder, H. W., Jr. (1982) *Virology* **117,** 165–172
49. Yu, K.-T., and Czech, M. P. (1984) *J. Biol. Chem.* **259,** 5277–5286
50. Erneux, C., Cohen, S., and Garbers, D. L. (1983) *J. Biol. Chem.* **258,** 4137–4142
51. Carpenter, G., King, L., and Cohen, S. (1979) *J. Biol. Chem.* **254,** 4884–4891
52. Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W., and Osathanondh, R. (1982) *J. Biol. Chem.* **257,** 15162–15166
53. Pike, L. J., Gallis, B., Casnellie, J. E., Bornstein, P., and Krebs, E. G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 1443–1447
54. Flockhart, D. A., and Corbin, J. D. (1982) *CRC Crit. Rev. Biochem.* **12,** 133–186
55. Collett, M. S., Wells, S. K., and Purchio, A. F. (1983) *Virology* **128,** 285–297
56. Cohen, S., Ushiro, H., Stoscheck, C., and Chinkers, M. (1982) *J. Biol. Chem.* **257,** 1523–1531
57. Purchio, A. F., Wells, S. K., and Collett, M. C. (1983) *Mol. Cell. Biol.* **3,** 1589–1597
58. Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M., and Cobb, M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3237–3240
59. Adkins, B., and Hunter, T. (1982) *Mol. Cell. Biol.* **2,** 890–896
60. Sefton, B. M., Hunter, T., and Raschke, W. C. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 1552–1556
61. Reynolds, F. H., Jr., Oroszlan, S., and Stephenson, J. R. (1982) *J. Virol.* **44,** 1097–1101
62. Patschinsky, T., Hunter, T., Esch, F. S., Cooper, J. A., and Sefton, B. M. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 973–977
63. Armstrong, R. N., Kondo, H., and Kaiser, E. T. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 722–725
64. McGrath, J. P., and Levinson, A. D. (1982) *Nature* **295,** 423–425
65. Gilmer, T. M., and Erikson, R. L. (1981) *Nature* **294,** 771–773
66. Wong, T. W., and Goldberg, A. R. (1983) *J. Biol. Chem.* **258,** 1022–1025
67. Wong, T. W., and Goldberg, A. R. (1984) *J. Biol. Chem.* **259,** 3127–3131
68. Hunter, T. (1982) *J. Biol. Chem.* **257,** 4843–4848
69. Nimmo, H. G., and Cohen, P. (1977) *Adv. Cyclic Nucleotide Res.* **8,** 126–146
70. Casnellie, J. E., and Krebs, E. G. (1984) *Adv. Enzyme Regul.* **22,** 501–515
71. Konopka, J. B., Davis, R. L., Watanabe, S. M., Ponticelli, A. S., Schiff-Maker, L., Rosenberg, N., and Witte, O. N. (1984) *J. Virol.* **51,** 223–232
72. Braun, S., Ghany, M. R., and Racker, E. (1983) *Anal. Biochem.* **135,** 369–378
73. Stadtmauer, L. A., and Rosen, O. M. (1983) *J. Biol. Chem.* **258,** 6682–6685
74. Shizuta, Y., Khandelwal, R. L., Maller, J. L., Vandenheede, J. R., and Krebs, E. G. (1977) *J. Biol. Chem.* **252,** 3408–3413
75. Shizuta, Y., Beavo, J. A., Bechtel, P. J., Hofmann, F., and Krebs, E. C. (1975) *J. Biol. Chem.* **250,** 6891–6896
76. Fukami, Y., and Lipmann, F. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1872–1876
77. Mroczkowski, B., Mosig, G., and Cohen, S. (1984) *Nature* **309,** 270–273
78. Sugimoto, Y., Whitman, M., Cantley, L. C., and Erikson, R. L. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 2117–2121
79. Macara, L. G., Marinetti, G. V., and Balduzzi, P. C. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 2728–2732
80. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 13341–13348
81. Brugge, J. S., and Darrow, D. (1984) *J. Biol. Chem.* **259,** 4550–4557
82. Levinson, A. D., Courneidge, S. A., and Bishop, J. M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 1624–1628
83. Henderson, L. E., Krutzschi, H. C., and Oroszlan, S. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 339–343
84. Schultz, A., and Oroszlan, S. (1984) *Virology* **133,** 431–437
85. Nishizuka, Y., Takai, Y., Hashimoto, E., Kishimoto, A., Kuroda, Y., Sakai, K., and Yamamura, H. (1979) *Mol. Cell. Biol.* **25,** 153–165
86. Maller, J., Foulkes, J. G., Erikson, E., and Baltimore, D. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 272–276